1-1987

3' Terminal Processing of Precursor tRNA Transcribed From a Drosophila Melanogaster Histidine Gene in a Cell-Free System

James P. Fulginiti
The College at Brockport

3' TERMINAL PROCESSING OF PRECURSOR tRNA TRANSCRIBED FROM A *DROSOPHILA MELANOGASTER* HISTIDINE GENE IN A CELL-FREE SYSTEM

A Thesis

Presented to the Faculty of the Department of Biological Sciences

of the State University of New York College at Brockport

in Partial Fulfillment for the Degree of

Master of Science

by

James P. Fulginiti

January, 1987

THESIS DEFENSE

Student Name James P. Fulginiti

APPROVED	NOT APPROVED	MASTER'S DEGREE ADVISORY COMMITTEE	
✓		L Kline Major Advisor	1/19/87 Date
✓		P. Mattfeld Fox Committee Member	1/19/87 Date
✓		J. Emory Morris Committee Member	1/19/87 Date

Edward E. Ayers 1987
Chairman, Graduate Committee

L Kline 1/19/87
Chairman, Department of Biological Sciences

ACKNOWLEDGEMENTS

I would like to thank Dr. Larry K. Kline for his guidance and supervision of this research project, and Drs. J. Emory Morris and P. Michael Fox for their helpful suggestions.

I wish also to thank Faith Rogers-Aubel, Diana Parker, John Till, Gregg Ublacker, and Nancy Sedita for their support during my research.

A special thanks to my wife Michaelene for her encouragement throughout my work at SUNY Brockport.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ABSTRACT

Transfer RNA biosynthesis is a complex process which includes trimmings at the 5' and 3' termini and nucleotide modification of the initial tRNA precursor. This research involves the detection and isolation of a 3' endonucleolytic activity from Schizosaccharomyces pombe.

tRNA precursors are obtained from a cell-free transcription system using (i) a Drosophila tRNA-histidine gene which contains a 35 base pair trailer sequence at its 3' terminus, and (ii) a crude yeast enzyme extract which can faithfully transcribe the gene and process the precursor transcripts. Transcription products are separated by means of polyacrylamide gel electrophoresis, visualized by autoradiography, and eluted from the gel. The tRNA precursors are then incubated with a Sc. pombe extract, electrophoresed and autoradiographed. The intact 35 base pair trailer sequence will serve as an indicator of the presence of the 3' endonuclease.

INTRODUCTION

The functional transfer RNA molecule is the result of the actions of a variety of enzymes: (i) polymerases which transcribe the tRNA gene into its precursor form; (ii) nucleases which trim the 5' and 3' flanking sequences of the precursor; and (iii) in eukaryotic systems, nucleotidyl transferase which adds the trinucleotide -CCA to the 3' terminus. Additional enzymes are needed in certain cases to splice dimeric tRNA transcripts, to remove intervening sequences, and to modify bases. The general outline of tRNA processing is shown in Figure 1.

Trimmings of the 5' and 3' flanking regions may occur by means of exonucleases or endonucleases. Exonucleases cleave the nucleotides from the ends of the precursor one at a time to produce the mature 5' and 3' termini. Endonucleases, in contrast, make single internal cleavages which generate the mature ends.

RNase P has been identified as an endonuclease which can cleave the precursor tRNA to produce the mature 5' terminus. RNase P appears to have universal recognition of precursor tRNAs.

Generation of the 3' terminus in eukaryotic systems may occur by means of the action of an endonuclease which has recently been detected in Xenopus laevis ovary extracts. It is unclear if this activity recognizes all tRNA species.

The mature tRNA with its attached amino acid serves as an adaptor molecule during protein synthesis, and is essential in the translation of the nucleotide sequences of messenger RNA into the amino acids which comprise proteins. Study of tRNA processing may yield additional information regarding the regulation of protein synthesis.

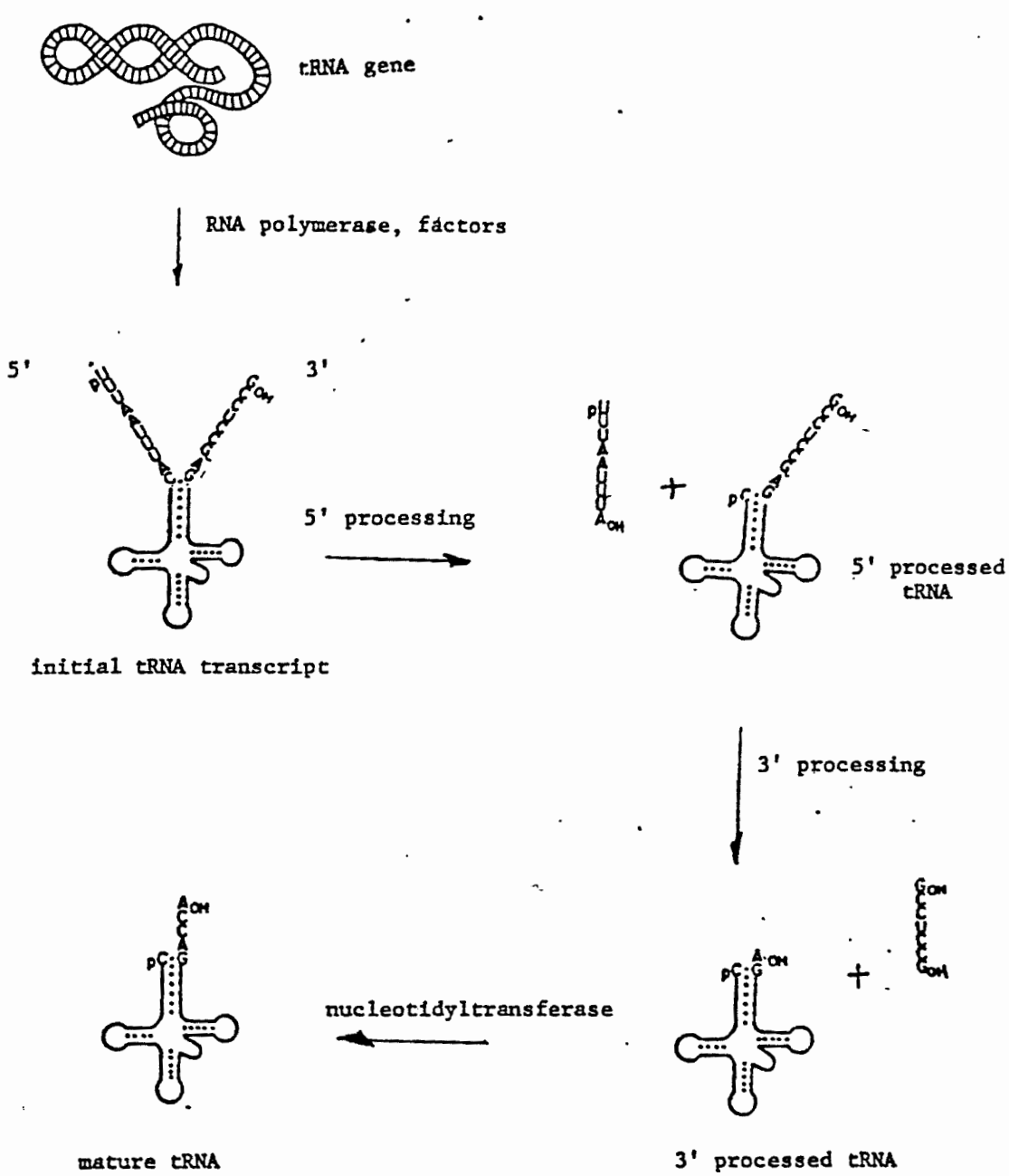

Figure 1 - Generalized schematic of tRNA gene transcription and processing of precursor tRNA. Base modifications and excision of intervening sequences occur prior to the addition of the 3' terminal -CCA.

STATEMENT OF PROBLEM

In order to study the enzymes involved in the processing of tRNA, it is necessary to isolate and identify tRNA precursors. *In vivo* processing is too rapid to allow for such isolation, and the intermediates are present only in low concentrations.

Sufficient amounts of substrate may be generated *in vitro* in two ways: (i) by using a cell-free system to transcribe a tRNA gene inserted into a plasmid, or (ii) by using a synthetic precursor produced after appropriate synthetic modifications of mature tRNA.

The *in vitro* transcription system was used in this research. A flow chart of the experimental design is given in Figure 2. A plasmid containing the tRNA-histidine gene from *Drosophila melanogaster* is transcribed using an enzyme preparation from *Saccharomyces cerevisiae*. Transcripts are radiolabeled with [α-^{32}P]GTP. This system was selected for the following reasons: (i) the 35 base pair trailer sequence of the *Drosophila* tRNA-his precursor is long enough to be detected if 3' processing is done by an endonuclease, (ii) the *S. cerevisiae* enzyme preparation contains both polymerases for transcription and nucleases for processing, and (iii) radiolabeling permits visualization of transcripts by means of autoradiography following separation of transcripts by polyacrylamide gel electrophoresis.

Once sufficient substrate has been produced using the cell-free transcription system described, the transcripts will be treated with an enzyme preparation from *Schizosaccharomyces pombe*. Further processing of the transcripts will occur. Detection of the intact 35 base pair trailer sequence will serve as an indicator of the presence of the 3' endonuclease.

The goals of this research project are summarized as follows:

(i) to obtain sufficient amounts of tRNA substrate for processing studies by means of a cell-free transcription system, and if possible

(ii) to use these substrates to detect a 3' endonuclease in *Sc. pombe*.

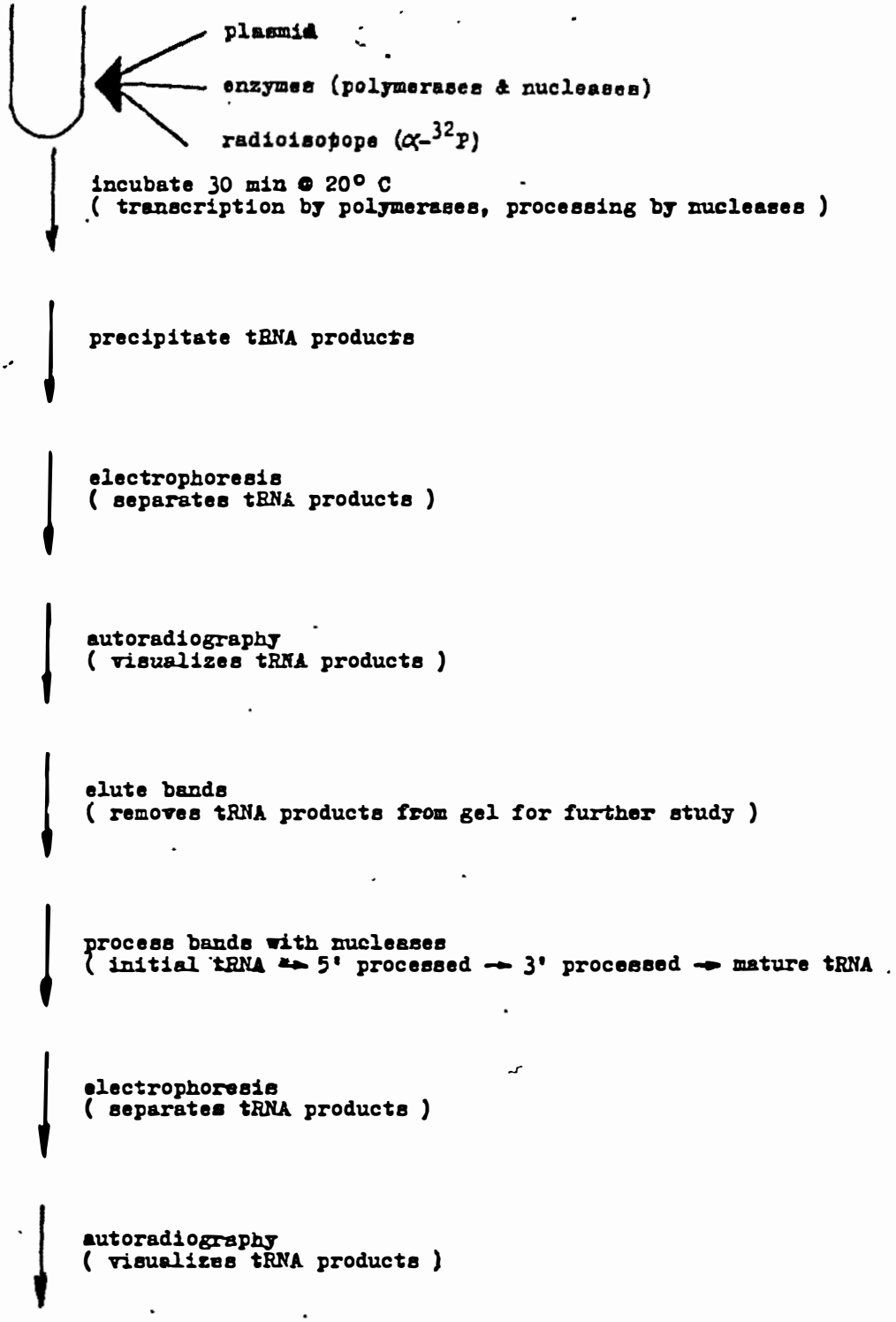

Figure 2 - Flow chart of experimental design using <u>in vitro</u> cell-free transcription of tRNA His gene and processing of tRNA precursors to detect 3' endonuclease.

LITERATURE REVIEW

Transfer RNA genes are transcribed into precursor molecules which are larger than the mature tRNA. Additional nucleotide sequences located on the 5' and 3' ends must be removed, intervening sequences if present are excised, and certain base modifications occur to produce the functional tRNA molecule. The series of reactions which converts the initial transcript to the mature tRNA is referred to collectively as tRNA processing.

Processing pathways may be studied using mutants deficient in processing enzymes. Mutants in *E. coli*, yeasts, and bacteriophages can be isolated using classical genetics procedures, but these procedures are not applicable to the study of tRNA processing in higher eukaryotes (Hopper 1984). Using recombinant DNA methods, eukaryotic tRNA genes are inserted into vectors and transcribed *in vitro* by means of cell-free systems in order to produce precursor tRNAs for processing studies (Deutscher 1984).

Processing events vary by species and by tRNA gene arrangement. In eukaryotic systems, tRNA genes are predominantly found in monomeric forms. Prokaryotic tRNA genes, in contrast, show a higher frequency of di- and multimeric arrangements. This variation in gene arrangement results in different types of initial tRNA transcripts (Sharp et al 1985). Multimeric precursors are cleaved by endonucleases such as RNase P2 to generate smaller fragments which are then subject to further 5' and 3' processing (Schedl et al 1976).

The mature 5' terminus is formed by the endonuclease RNase P, found in both prokaryotes and eukaryotes (Kline et al 1981; Altman et al 1980).

It is noteworthy that RNase P is a ribonucleoprotein consisting of a low molecular weight protein and an RNA of ca. 375 nucleotides. Both components are necessary for activity: the RNA is most likely needed for correct positioning of the precursor tRNA at the enzyme's active site (Kole and Altman 1979). Studies in eukaryotic systems have demonstrated that 5' processing by RNase P precedes processing of the 3' terminus (Frendewey et al 1985).

An endonuclease which universally processes the 3' terminus in eukaryotes and prokaryotes has not yet been detected. A variety of exonucleases have been shown to be involved in the generation of the 3' terminus in prokaryotes (Kole and Altman 1982). Experiments performed in eukaryotic systems by Garber and Gage suggest that an endonucleolytic activity present in a Xenopus laevis oocyte extract was responsible for the cleavage of the 3' trailer sequence (1979). Additional studies have yielded results which support the involvement of a 3' processing endonuclease in eukaryotic systems (Solari and Deutscher 1983; Adeniyi-Jones et al 1984). Purification and characterization of an endonuclease from X. laevis ovaries which processes the 3' terminus of pre-tRNA-met has been reported by Castaño et al (1985).

Two enzymatic activities are required for the removal of introns present in eukaryotic tRNA genes. An endonuclease acts first to excise the intron; a ligase then rejoins the free ends of the exons (Peebles et al 1983; Greer et al 1983).

Base modifications, such as pseudouridine and thiouridine, are the result of a variety of enzymes. These modifications appear to serve as recognition sites for processing enzymes (Kline and Söll 1982).

Generation of the mature 3' terminus ending in the trinucleotide

sequence -CCA differs between prokaryotes and eukaryotes. In prokaryotes, the -CCA sequence is encoded within the tRNA gene. Eukaryotic tRNA genes, however, do not code for this sequence. The triplet is added post-transcriptionally by the enzyme tRNA nucleotidyl transferase. Because the terminal A is subject to turn over, this enzyme is also needed to maintain the -CCA sequence (Lewin 1985).

One additional processing event is necessary in the production of the mature tRNA-his, which is one nucleotide longer at its 5' end than all other tRNA species. A 5' terminal guanylate residue, not encoded by the tRNA-his gene, is added post-transcriptionally by an ATP-requiring enzymatic activity. Nucleotide addition at the 5' end has not been found in other tRNAs (Cooley et al 1982).

A multiplicity of enzymes capable of acting on tRNA precursor molecules has been detected in E. coli. The degree to which they have been purified and characterized varies greatly. Table I lists a number of exo- and endonucleases believed to process tRNA transcripts in E. coli (Deutscher 1985).

Why the mature tRNA is not transcribed directly from the gene (i.e. why is processing necessary) is not clear. Regulation of genes coding for processing enzymes will affect the number of amino-acylated tRNAs available in the translation of mRNA into protein. How these genes are regulated is also unclear (Frendewey 1985; Sharp et al 1985).

In vitro studies of the processing of eukaryotic tRNA have been facilitated by recombinant DNA techniques. Eukaryotic tRNA gene sequences can be inserted into bacterial plasmids to produce sufficient amounts of substrate for processing studies. Plasmid pYM10.3 was constructed from pBR322 and a 1.1 kb fragment of Drosophila DNA shown to

contain the tRNA-his gene through its ability to hybridize to histidine tRNA. pBR322 is a double-stranded, circular DNA containing tetracycline and ampicillin resistance genes. Both Bam HI and Hind III restriction endonucleases have unique recognition sequences within the tetracycline resistance gene (see Figure 3). pBR322 was digested with these two enzymes, thereby (i) excising a fragment of ca. 345 base pairs, and (ii) destroying tetracycline resistance (Cooley et al 1982; Cooley et al 1984).

The 1.1 kb fragment containing the tRNA-his gene was inserted into the Bam HI/Hind III-digested plasmid. E. coli cells were subsequently transformed with the plasmid construct, and plated onto selective media. Those cells showing an inability to grow on tet+ medium, but able to grow on amp+ medium are designated as transformants.

The initial transcript of Drosophila tRNA-his gene is shown in figure 4. It contains a 35 bp 3' trailer sequence which is long enough to be detected if 3' terminal processing is performed by an endonuclease (Frendewey et al 1985).

Table I E. coli RNases involved in tRNA processing. (Deutscher 1985)

RNase	Type	Substrate	Product
P	endo	pre-tRNA (5' end)	5'-P tRNA
BN	exo	pre-tRNA (3' end)	tRNA (incomplete -CCA end)
P2	endo	multimeric pre-tRNA	smaller pre-tRNA
PC	endo	pre-tRNA	smaller pre-tRNA
D	exo	pre-tRNA (3' end)	5'-P mono NT and tRNA-CC
T	exo	tRNA-CCA (3' end)	5'-P mono NT and tRNA-CC

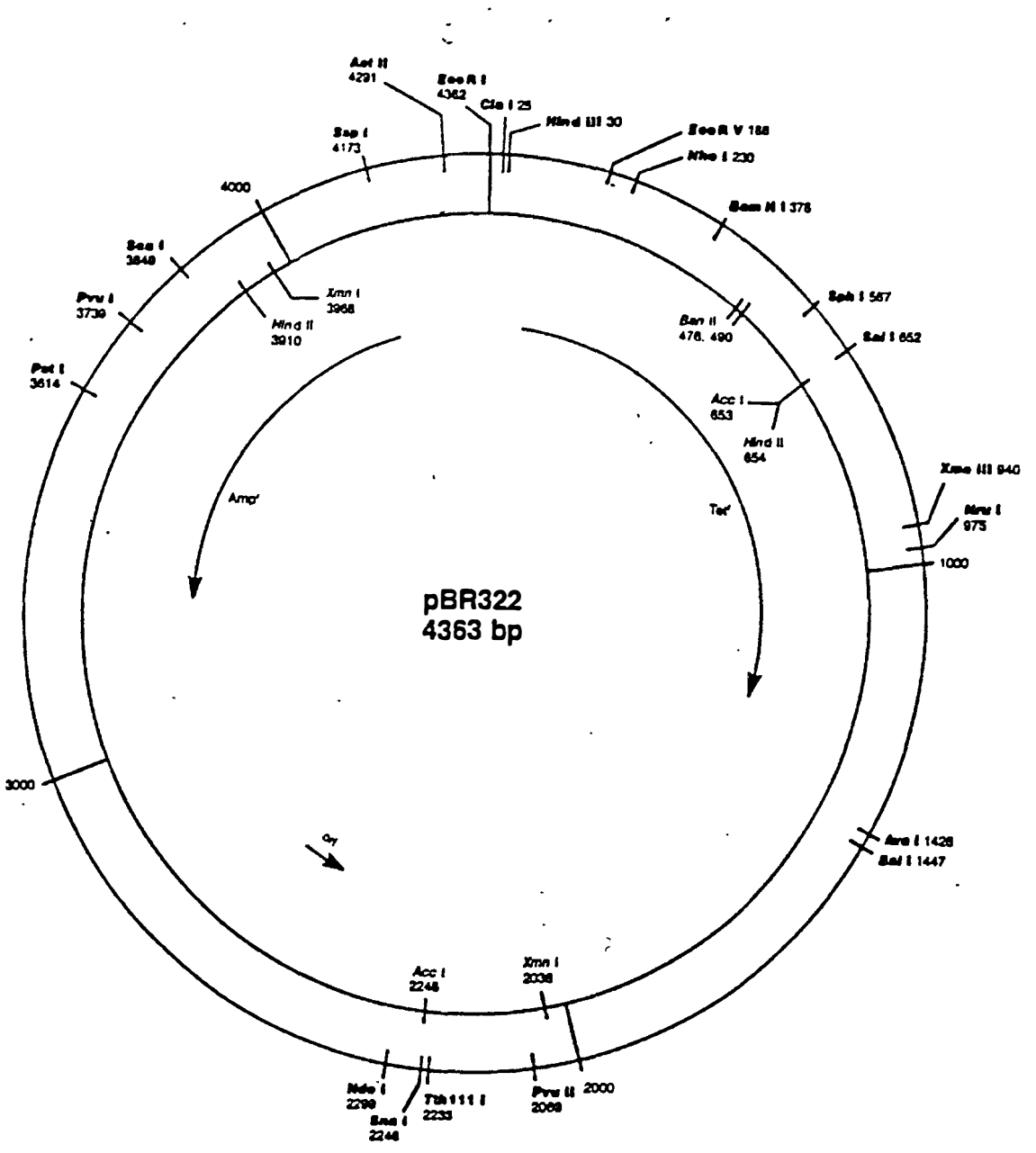

Figure 3 - Restriction map of pBR322. The fragment between the Bam HI and Hind III sites was excised first, the Drosophila tRNA-his gene was then inserted, thereby destroying tetracycline resistance (BRL 1985).

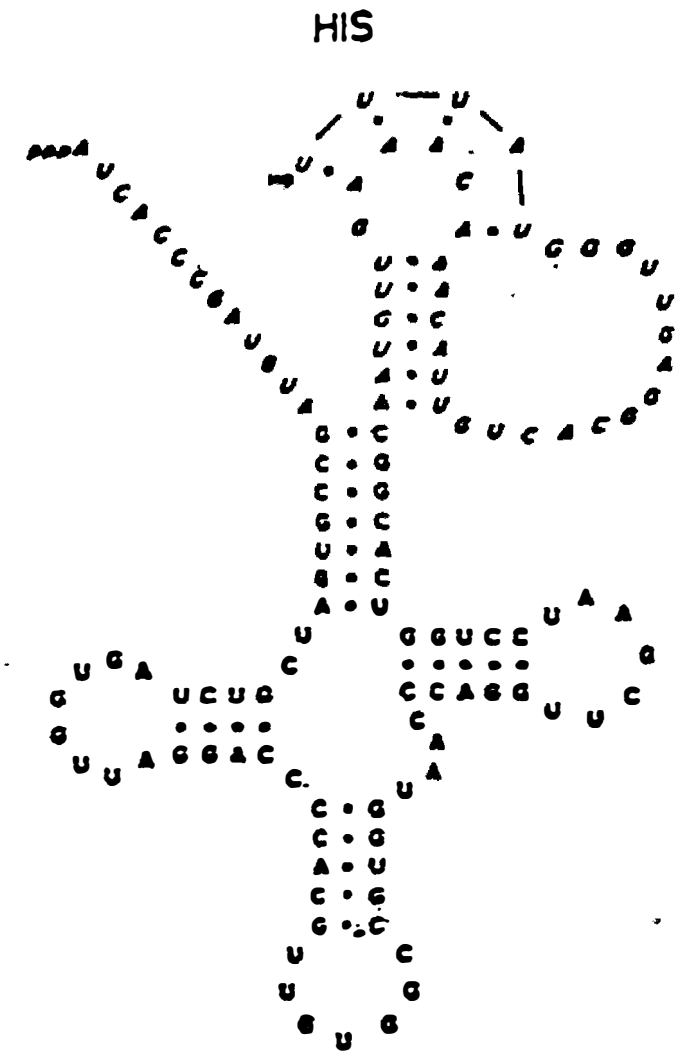

Figure 4 - Possible secondary structure of the initial tRNA-his transcript. Bases in italics are present in the precursor only (Frendewey et al 1985).

MATERIALS AND METHODS

Materials

α-^{32}P-labeled ribonucleoside triphosphates were obtained from New England Nuclear, Boston, Massachusetts. Kodak X-Omat AR film was used for autoradiography of gels. Reagent grade chemicals from commercial suppliers were used to prepare all solutions.

Plasmid DNA

Plasmid Amplification - pYM10.3 (also designated p48His) was donated by Dieter Söll, Yale University. Escherichia coli strain HB101 had been previously transformed with pYM10.3 and stored at -70 C in a 1:1 dilution with dimethyl sulfoxide (DMSO). One milliliter of this inoculum was added to 10 ml of Luria-Bertani (LB) medium [Bacto-tryptone 10 g/l, Bacto-yeast extract 5 g/l, NaCl 10 g/l, adjusted to pH 7.5 with NaOH] containing 20 ul ampicillin (50 ug/ml). The culture was incubated overnight at 37°C. Forty milliliters of LB medium containing 80 ul ampicillin (50 ug/ml) was inoculated with 0.1 ml of the overnight culture, and incubated with shaking at 37°C for 4-5 hr until late log phase was reached ($A_{650} \cong 0.6$). Twenty-five milliliters of the late log culture was used to inoculate 500 ml of LB medium containing 1 ml ampicillin (50 ug/ml), and incubated with shaking for 2.5 hr at 37°C ($A_{650} \cong 0.4$). To this culture was added 2.5 ml of chloramphenicol (3.4 mg/ml in ethanol) to a final concentration of 170 ug chloramphenicol per milliliter. The culture was now incubated with shaking for 12-16 hr at 37°C (Maniatis et al 1984).

Isolation of Plasmid DNA - Bacterial cells from the amplification procedure were harvested by centrifugation at 5000g for 5 min at 4°C using the Sorvall GSA rotor. The supernatant was discarded, and the cells were washed in 40 ml of ice-cold STE [0.1 M NaCl, 10 mM Tris Cl (pH 7.8), 1 mM EDTA]. Ten milliliters of an ice-cold solution of 10% sucrose in 50 mM Tris Cl (pH 8.0) was used to resuspend the pellet. The cells were then transferred to a 40 ml centrifuge tube, and 2 ml of freshly-prepared lysozyme (10 mg/ml in 0.25 M Tris Cl, pH 8.0) was added. Eight milliliters of 0.25 M EDTA was immediately added. The solution was mixed by inverting the tube and placed on ice at 0°C for 10 min. Four milliliters of 10% SDS was added; the solution was mixed quickly but gently with a glass rod to disperse the SDS evenly. To this supension was added 6 ml of 5 M NaCl (for a final concentration of 1 M NaCl) with gentle mixing with a glass rod. The mixture was placed on ice for at least 1 hr, and then centrifuged in Oakridge tubes using the Beckman Ti-50 rotor for 30 min at 30,000 rpm at 4°C. The supernatant was extracted twice with 1:1 phenol/chloroform (v/v) saturated with 50 mM Tris Cl (pH 8.0), and once with chloroform saturated with 50 mM Tris Cl (pH 8.0). Aqueous and organic phases were separated by centrifugation in the SS-34 rotor for 5 min at 8000g and 4°C. The aqueous phase was transferred to a 250 ml flask, to which was added two volumes of ethanol. The solution was mixed and placed in the freezer at -20°C overnight. Centrifugation in the Sorvall using the SS-34 rotor for 15 min at 6000g and 4°C produced a pellet of nucleic acids which was washed with 95% ethanol at room temperature, and inverted to dry. The DNA pellet was then dissolved in 8 ml of TE (pH 8.0) (Maniatis et al 1984).

The plasmid DNA was separated from the bacterial chromosomes by centrifugation to equilibrium in a cesium chloride-ethidium bromide gradient. Solid cesium chloride was added to the DNA solution (1 g per milliliter). The solution was gently mixed to dissolve all of the salt. For every 10 ml of CsCl solution, 0.8 ml of a solution of ethidium bromide (10 mg/ml in H_2O) was added and mixed thoroughly. The refractive index of the solution should be 1.3860, corresponding to a density of 1.55 g/ml and an ethidium bromide final concentration of ca. 600 ug/ml. Cellulose nitrate tubes were used to centrifuge the solution in the Beckman Ti-50 rotor. The solution was then centrifuged at 40,000 rpm for 36 hr at 20°C (Maniatis et al 1984).

Centrifugation separates the DNA into two bands which can be visualized using UV light: an upper band of linear bacterial chromosomes and fragments, and a lower band of closed circular plasmid DNA. The tubes were clamped to a ring stand and punctured with a syringe (#21 hypodermic needle) at the level of the plasmid DNA, taking care to remove as much of this band as possible without disturbing the gradient. The plasmid DNA was then transferred to a 15 ml plastic assay tube (Maniatis et al 1984).

Ethidium bromide was removed by adding an equal volume of 1-n-butanol saturated with H_2O, and mixed thoroughly by inverting the tube. The aqueous and organic phases were separated using a clinical centrifuge, 1500g for 3 min. The upper organic phase containing pink ethidium bromide was discarded. The lower aqueous phase was extracted an additional 7 times with 1-n-butanol saturated with H_2O, or until all the pink color disappeared from the aqueous phase. Cesium chloride was removed from the plasmid DNA by dialyzing the aqueous solution against

several changes of TE buffer (pH 8.0) (Maniatis et al 1984).

Quantification of Plasmid DNA - The amount of plasmid DNA obtained was determined by use of the Beckman spectrophotometer, (1 A_{260} = 50 ug/ml). An undiluted portion of the sample was measured. The entire sample was then lyophilized and resuspended in an amount of distilled water to give the concentration of plasmid DNA required for the transcription assay. The DNA was stored at -20°C and/or at 4°C for immediate use.

Saccharomyces cerevisiae

S. cerevisiae 208-12 (α trp-1 pep 4-3), a proteinase- and ribonuclease- deficient strain, was donated by Dr. Dieter Söll, Yale University.

Growth of Cells - Cells were grown in YEPD medium [1% w/v yeast extract, 2% w/v Bacto-peptone, 2% w/v dextrose] (Maniatis et al 1984). An initial culture was started by inoculating 100 ml of YEPD with an isolated colony from an agar plate, and was allowed to incubate at 30°C overnight with shaking. One to five milliliters of the overnight culture was transferred to another 100 ml of YEPD. At 1 hr intervals, the density of the culture was measured by absorbance at 650 nm in order to determine the doubling time. Two flasks, each containing 1500 ml YEPD, were inoculated with an amount from this culture such that A_{650} the next morning would be ca. 3.

Preparation of S. cerevisiae Enzyme Extract - An enzyme extract used in both transcription and processing assays was prepared from cells using the method of Klekamp and Weil (1982) with modifications by Schaak and Soll (1985). Cells from the 1500 ml cultures were put on ice, harvested using the Sorvall GSA rotor, 4000g for 5 min at 4°C, and then washed twice in cold distilled water. Cells should be creamy white, with no blackish residue. Eight grams of yeast cells and eight grams of 0.5 mm glass beads were placed in the 30 ml chamber of the Bead Beater Cell Disrupter (Biospec Products, Bartlesville, Oklahoma). The chamber was then completely filled with solubilization buffer [200 mM Tris Cl (pH 8.1), 10% v/v glycerol, 10 mM MgCl, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF)], noting the amount added. The assembled chamber was placed in a jacket containing a $CaCl_2$-ice slurry. Cells were lysed at 4°C using 30 second bursts for a total of 3 min, allowing 2 min between bursts for cooling (at no time should the chamber temperature exceed 10°-12°C).

The chamber was emptied into 40 ml centrifugation tubes and rinsed with 5 ml solubilization buffer. Centrifugation in the Sorvall SS-34 rotor at 7000 rpm for 10 min recovered the lysate. PMSF and β-mercaptoethanol were each added to the lysate to 1 mM and 10 mM final concentrations respectively. A 3.7 M ammonium sulfate solution (pH 7.9) was then added dropwise with gentle stirring to the lysate to a final concentration of 0.4 M. The mixture was allowed to sit on ice for 10-15 min, and then centrifuged in Oakridge tubes in the Ti-50 rotor at 40,000 rpm for 60 min at 4°C. The supernatant was pipetted into a graduated

cylinder, noting its volume, and then transferred to a small beaker. Finely-ground ammonium sulfate (0.35 g per ml supernatant) was added to the supernatant with stirring over a period of 10-15 min. The mixture continued to stir an additional 30 min to completely dissolve the salt and to precipitate the proteins. Centrifugation in the SS-34 rotor at 12,000 rpm for 15 min pelleted the protein precipitate, which was then resuspended in 1-2 ml of Buffer C [20 mM HEPES (pH 7.9), 20% v/v glycerol, 0.2 M EDTA, 10 mM β-mercaptoethanol, 0.1 M PMSF]. The protein extract was dialyzed overnight at 4°C against 1 liter of Buffer C containing 100 mM NaCl, and stored at -70°C in 50 ul aliquots.

Schizosaccharomyces pombe

Cell Culture - An isolated colony from an agar plate was used to inoculate 100 ml of medium [0.5% yeast extract, 3% dextrose] and placed in the incubator-shaker at 30°C overnight. Ten milliliters of the overnight culture was transferred to a second 100 ml of medium. The doubling time of the cells was determined by measuring the optical density at 650 nm at hourly intervals. Two flasks, each containing 1500 ml of medium, were inoculated with an amount of this culture such that the A_{650} the following morning would be approximately 3. Cells were harvested in the Sorvall GSA rotor at 4000 rpm for 5 min, and either used fresh for enzyme extraction or stored in 8 g aliquots at -70°C.

Enzyme Extraction - Eight grams of cells and eight grams of 50 um glass beads were placed in the 30 ml chamber of the Bead Beater Cell

Disrupter, which was then completely filled with buffer [0.05 M Tris Cl (pH 8.0), 0.01 M β-mercaptoethanol, 10% v/v glycerol, and 0.1 M KCl]. The unit was then assembled with a calcuim chloride slurry in the jacket. Cells were lysed using 30 sec bursts with 2 min cooling time between bursts. The temperature in the chamber should not exceed 10°-12°C. The homogenate was poured into a small beaker to allow the beads to settle, and then into a 40 ml centrifuge tube. Chamber and beads were rinsed with 10-15 ml buffer. Centrifugation in the Sorvall SS-34 rotor at 4000 rpm for 5 min removed the glass beads and whole cells. The supernatant was first transferred to a 40 ml tube and centrifuged at 12,000 rpm for 10 min, then to Oakridge tubes for centrifugation in the Ti-50 rotor at 40,000 rpm for 90 min at 4°C. The enzyme extract was dialyzed overnight against buffer [0.05 M Tris Cl (pH 8), 0.01 M β-mercaptoethanol, 10% v/v glycerol]. The extract was stored in aliquots at -70°C and/or used fresh in the processing assay.

Methods

Transcription Assay - Transcription assays were performed at 20 C in a final reaction volume (50 ul) of 12 mM Hepes (pH 7.9), 150 mM NaCl, 10mM MgCl , 12% v/v glycerol, 6 mM dithiotreitol, 0.6 mM each of CTP and UTP, 1.2 mM ATP, 7.5-15 uCi [α - ^{32}P]GTP (specific activity 800 Ci/mmol), and 25 ug/ml of plasmid DNA, and 1-5 ul of the *S. cerevisiae* enzyme extract (Klekamp and Weil, 1982; Schaak and Söll, 1985). Reaction time was 30-40 min. Transcription was terminated by the addition of 40 ul of a proteinase K solution (1 mg/ml in 0.5% SDS) and subsequent incubation for 30 min at 37°C. The reaction products were extracted by

adding 60 ul EBS buffer [300 mM NaCl, 1.0 mM EDTA, 10 ul of a phenol: chloroform: isoamyl alcohol solution (v/v/v 50:48:2) saturated with TE buffer]. Assay vials were vortexed and then centrifuged to separate the aqueous and organic phases. The aqueous phase was transferred to a clean vial, and the organic phase reextracted with an additional 100 ul EBS. Reaction products were precipitated in 2.5 volumes of absolute ethanol (625 ul) and placed in the freezer for 5-6 hr or overnight. Centrifugation in the Sorvall SS-24 rotor at 7000 rpm for 10 min recovered the tRNA precipitate, which was resuspended in 200 ul of distilled water. Reprecipation of the tRNA was accomplished by addition of 20 ul (0.1 volume) of 1 M sodium acetate (pH 5.4) and 500 ul absolute ethanol, and by placing the vials for 5-6 hr or overnight in the freezer. The tRNA precipitate was recovered by centifugation as noted above, and lyophilized to dryness. To prepare the tRNA for electrophoresis, 5 ul tracking buffer [7 M urea, 0.5 M Tris-borate electrophoresis buffer, 10 mM EDTA, 0.1% Xylene cyanol FF, and 0.1% Bromophenol blue] was added to each vial.

Polyacrylamide Gel Electrophoresis - The gel used to analyze tRNA substrates was 10% polyacrylamide in 8.3 M urea. Gels were prepared from 40% stock [380 g acrylamide and 20 g bisacrylamide per liter, powdered charcoal, then filtered], 10X TBE [10X = 121.2 g Tris Cl, 611.8 g boric acid, 7.4 g $Na_2EDTA \cdot 2H_2O$], and 50% w/v urea. The mixture was stirred over low heat until the urea dissolved, then brought to within 1% of final volume with distilled water. The solution was deaerated under vacuum. TEMED (N,N,N',N' - tetramethylethylene diamine) was added (30 ul

per 100 ml final volume), and finally a solution of ammonium persulfate [150 mg in 5 ml distilled H_2O] (1 ml per 100 ml final volume). The solution was mixed well and immediately poured into a preassembled mold to polymerize.

The polymerized gel was run in TBE prior to loading samples. For 2 mm x 15 cm gels, 300 V and 6-8 ma for 3 hr was applied. Larger gels (2 mm x 35 cm at 200 V and 8-10 ma) were used for overnight electrophoresis.

Autoradiography - Products of transcription and processing assays were visualized using autoradiography. Following electrophoresis, the back glass plate was removed, and the gel covered with plastic wrap. The position of both tracking dyes was marked. In the darkroom, the gel was covered with a 8 x 10 in sheet of Kodak X-Omat AR film. Exposure time for transcription was 3-24 hr; for processing assays 3-10 days. Film was placed in developer for 4 min, rinsed in water, and agitated in fixer 5-7 min.

Recovery of tRNA Substrates from Polyacrylamide Gels - Products of the transcription assay were recovered for further study. Using the exposed film as a template, the tRNA substrates were cut out from the gel. The approximate amount of radioactivity in each band was determined using Cerenkov counting. To the crushed gel bands was added 1 ml elution buffer [0.3 M NaCl, 10 mM Tris Cl (pH 7.5), 1 mM EDTA, 1% phenol]. Gels were then placed in the incubator shaker at 37°C overnight. The liquid was separated from the gel fragments by centrifuging the slurry through a 1 ml pipette tip plugged with glass wool (Schlief and Wensink, 1981).

The crushed gel was reeluted with 0.5 ml elution buffer for several hours more. The eluted products were then counted Čerenkov to determine the recovery efficiency.

Several ethanol precipitations were used to remove contaminants from the tRNA products. The precipitate was lyophilized to dryness, and resuspended in 50 ul distilled water. A 2-5 ul sample was pipetted onto Whatman 3 mm disks and counted in Omnifluor to determine total ^{32}P counts. A minimum of 1000 counts was used in the processing assay.

Processing Assays - Total volumes used in processing assays were 50 ul, of which 25 ul was 2X buffer, 10 ul was enzyme extracts from S. cerevesiae and/or Sc. pombe, and eluted tRNA products. The assays were incubated at 37 C for 30 min, followed by two extractions with 100 ul EBS buffer in 100 ul phenol: chloroform: isoamyl alcohol saturated with TBE. Carrier tRNA (2 ul of a 4 mg/ml solution) was added during the first extraction. Products of the processing assay were ethanol precipitated twice, lyophilized to dryness, resuspended in 5 ul loading buffer, and subjected to electrophoresis and autoradiography.

RESULTS

Plasmid DNA

The plasmid yield from the 1000 ml culture was determined by measuring 1 ml of the undiluted sample for absorbance at 260 nm, where 1 A_{260} is equivalent to 50 ug of DNA. A reading of 0.823 was obtained, indicating a concentration of 41.2 ug/mg. Total volume of the plasmid prep was 10 ml, thus a yield of 412 ug DNA from the 1000 ml culture. This compares to the expected yield from such a culture of about 2 mg (Maniatis et al 1984). The lower yield may have been a result of incomplete extraction of the plasmid band from the cesium chloride gradient, and from losses of material through procedural handling.

The A_{260}/A_{280} ratio can be utilized to determine the presence of protein or RNA contaminants. A ratio of 1.8 indicates pure DNA. A higher value indicates the presence of RNA, a lower value indicates the presence of protein. The A_{280} of the 1 ml undiluted sample was 0.431, giving a ratio of 1.91. This indicates the presence of RNA contaminants (Maniatis et al 1984), which may have resulted during extraction of the plasmid DNA from the centrifuge tube.

S. cerevisiae enzyme extract

The preparation of the S. cerevisiae enzyme extract used for transcription was done on five occassions. No active transcription complex was obtained, as determined by its ability to transcribe pYM10.3. The following parameters during the preparations were closely considered: cell culture density, color of cells (no blackishness), temperature,

time, presence of air bubbles while rupturing cells in the Bead Beater chamber, and amount and rate of addition of ammonium sulfate to precipitate proteins. The reason for lack of activity in the enzyme extract is unclear. Protein factors necessary for an active transcription complex may have failed to precipitate in the ammonium sulfate cut, or a contaminant may have inhibited enzyme activity.

Transcriptions using plasmid DNA and S. cerevisiae extract

An active S. cerevisiae enzyme extact prepared by Diana Parker on October 15, 1985, and stored at -70° C, was used in the transcription and processing studies.

A total of 24 transcription experiments was performed, using conditions described by Klekamp and Weil (1982), and Schaack and Söll (1985). Because the enzyme extract contains polymerases and nucleases, both transcription of the plasmid DNA and processing of the initial tRNA transcript occur. Figure 5 (A) depicts the expected gel pattern following treatment with the S. cerevisiae extract. Figure 6 shows the actual gel pattern obtained by means of autoradiography. Transcription and subsequent processing occur only in the presence of the enzyme extract.

As processing occurs, the intial transcript becomes shorter, losing sequences at its 5' and 3' ends. The variation in size will cause the processed species to migrate at different rates during electrophoresis. The large initial transcript will migrate the most slowly, and the mature tRNA will migrate faster than the other processed species.

Bands assumed to represent the initial transcript, 5' processed

tRNA, 3' processed tRNA, and mature RNA are clearly visible. The absence of the 3' trailer is most likely due to rapid degradation by contaminating exonucleases. Analysis of the assumed species by sequencing of terminal groups would be necessary in order to confirm the identity of the bands.

Recovery of tRNA Products from Gels

The autoradiograph was used to determine the position of the desired tRNA products on the gel. Bands were cut out and placed in microfuge tubes. Amount of ^{32}P incorporated into transcripts was approximated using whole gel Cerenkov counting on the ^{3}H channel of the Packard liquid scintillation counter. The conversion of 1 Cerenkov count to 2 ^{32}P counts was used, (e.g. 10,000 Cerenkov counts = 20,000 ^{32}P counts). The Cerenkov counts were used to determine efficency of recovery of the tRNA products in subsequent elution procedures.

Transfer RNA products were eluted in buffer as previously described. Cerenkov counting was done on the elution buffer following the first and second elutions to determine percent recovery of labeled products from the gel. Counts were adjusted by subtracting 5% per day of the whole gel counts to account for the decay of ^{32}P. A recovery of between 80% and 90% was typically obtained. A sample calculation, using data from March 12, 1986, is given in Table II.

Following precipitation and resuspension of the recovered tRNA, a small aliquot of material was counted on Whatman disks in Omnifluor using the ^{32}P channel.

Table III gives the results of ^{32}P counting of recovered material

and compares it to approximations obtained from Cerenkov counts. The low actual amounts recovered were determined to be partially due to incomplete precipitation of the tRNA from ethanol.

Processing Studies

Following elution of the various radiolabeled tRNA species from the gel, studies were conducted to detect the presence of a 3' processing endonuclease in enyzyme extracts from S. cerevisiae and Sc. pombe. Figure 5(B) shows the expected gel patterns following treatment of the eluted tRNA transcription products with the enzyme extract. Twelve processing experiments were performed, in which enzyme concentration, incubation time and temperature, and requirement for ATP were considered. As can be seen in Figure 7, no processing of the eluted material was observed. It was at first suspected that the eluted tRNA was contaminated with salts, urea, or another substance from the elution procedure which would inhibit enzyme activity. Multiple ethanol precipitations and washings of the pelleted tRNA were done to remove salts.

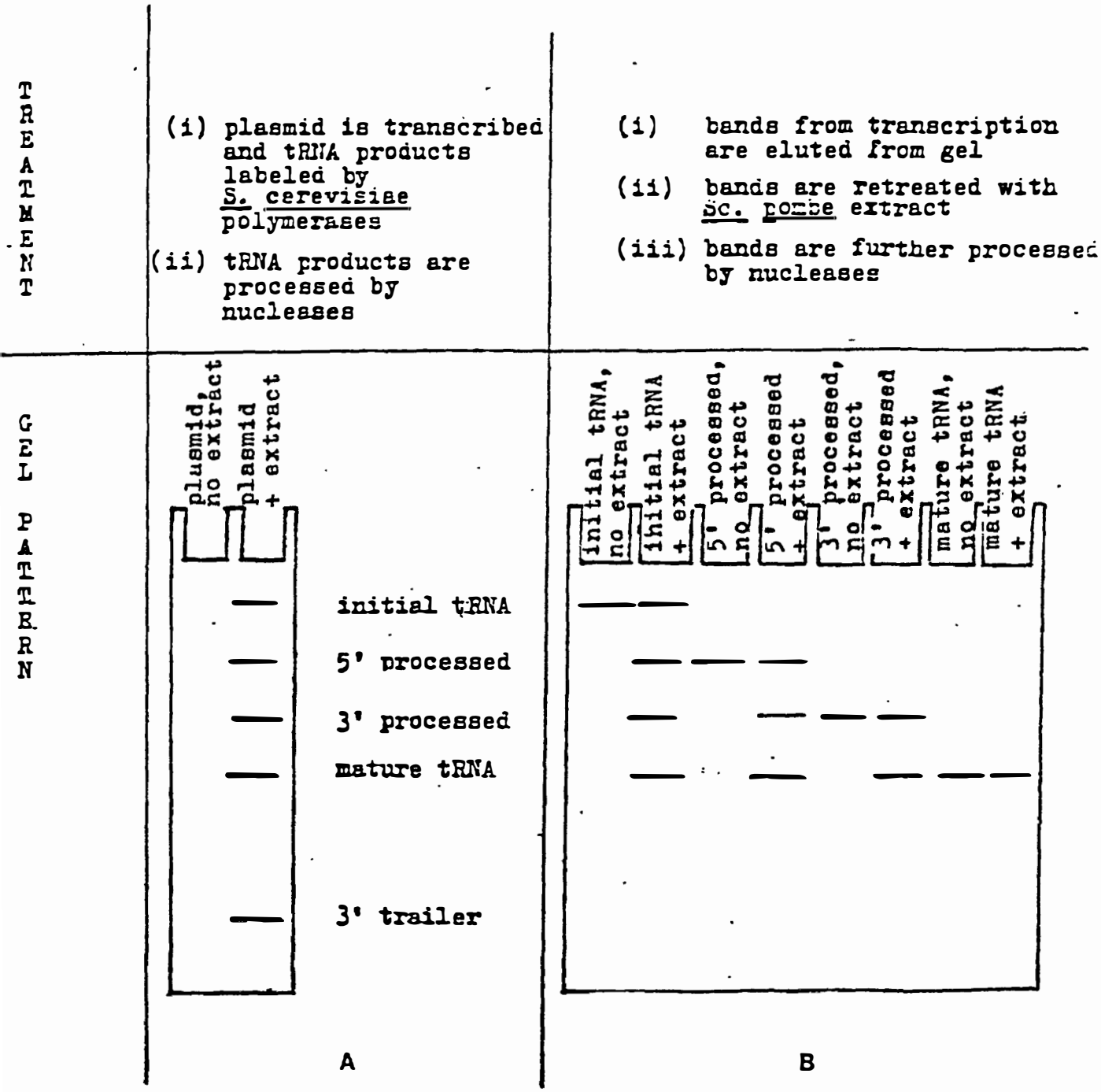

Figure 5 - Expected gel patterns following (A) initial transcription and processing by S. cerevisiae enzyme preparation and (B) subsequent processing by Sc. pombe nucleases.

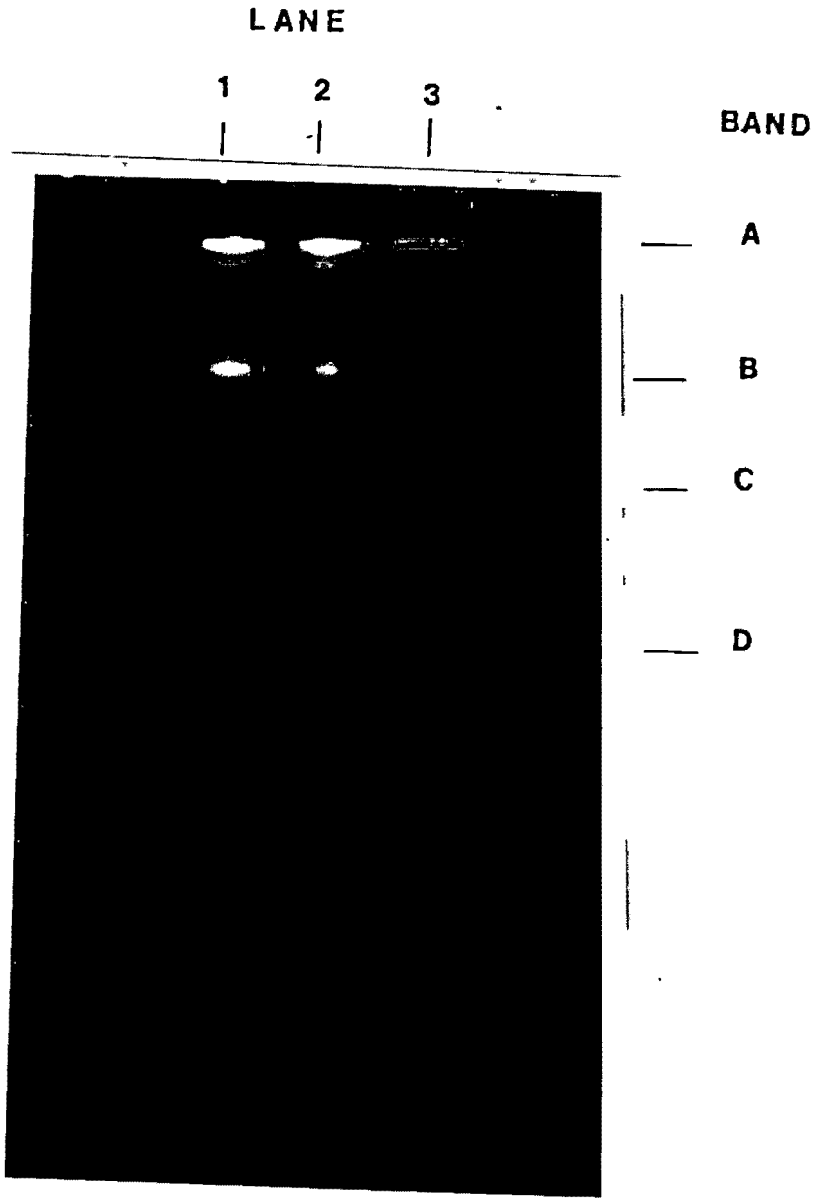

Figure 6 - Autoradiograph of Transcription of pYM10.3

Lane 3, no DNA; Lanes 1 & 2, 250 ul/ml DNA.

Tentative identification of bands:

Band A, initial tRNA transcript; Band B,

5' processed tRNA; Band C, 3' processed tRNA;

Band D, mature tRNA. Further analysis would

be required to confirm identification of bands.

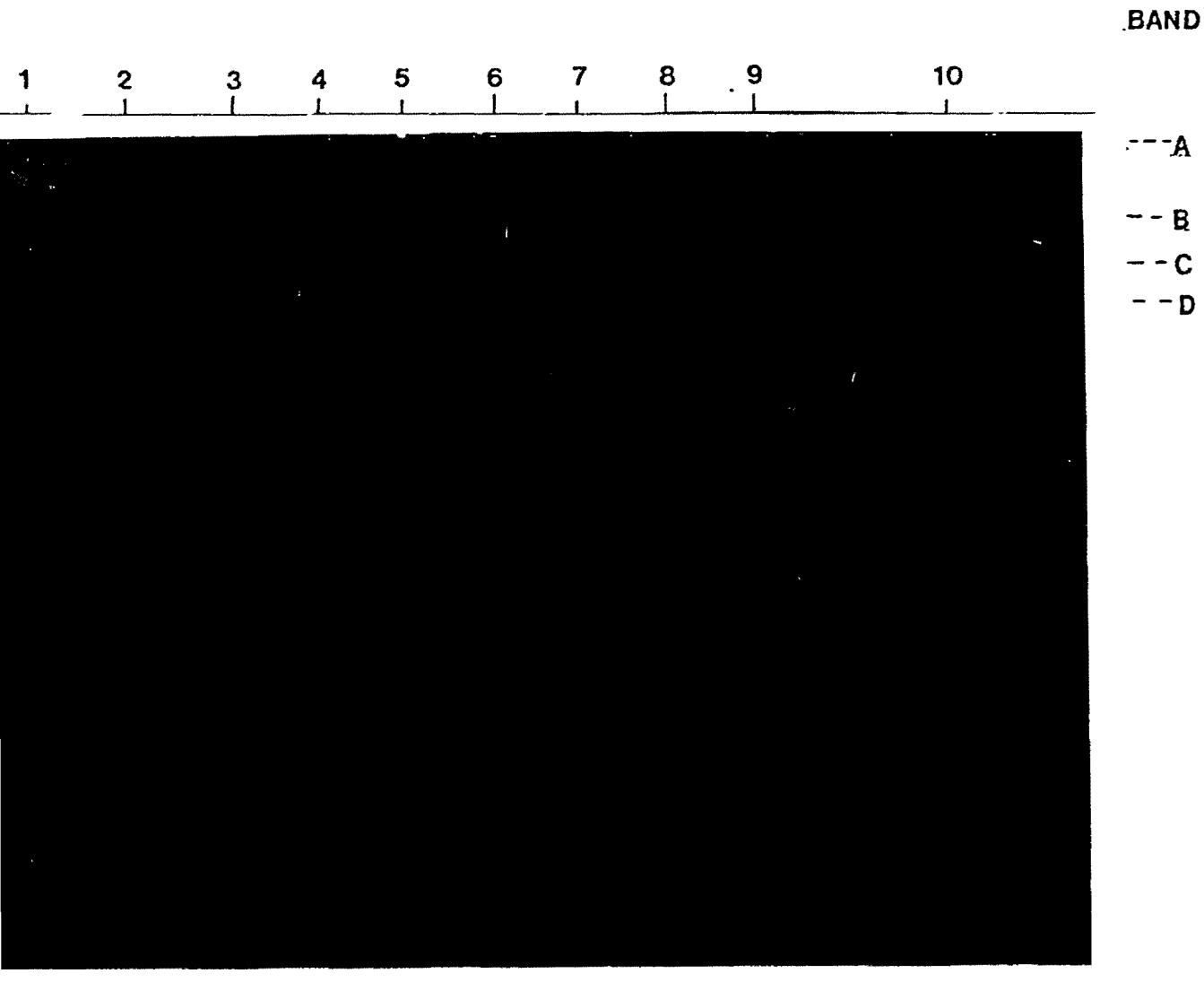

Figure 7 - Autoradiograph of processing of eluted tRNA transcripts.

Lanes 1,4,7,10: no enzyme extract added

Lanes 2,5,8: S. cerevisiae extract added

Lanes 3,6,9: Sc. pombe extract added,

Bands A, B, C, D, are the same as those tentatively identified in Figure 6.

Band	Whole Gel Counts-actual March 12, 1986	Whole Gel Counts (calc. -5%/day) March 13, 1986	Elution Buffer Counts-actual March 13, 1986	% Recovered	Estimated $^{32}P_{\wedge}$ Counts 2X Cerenkov March 13, 1986
A	31,113	29,557	26,093	88.3	52,186
B	19,227	18,266	15,310	83.8	30,620
C	17,534	16,657	14,681	88.1	29,362
D	25,799	24,509	20,432	83.4	40,864

TABLE III. ^{32}P Counting of tRNA Products

Band	^{32}P Counts of 2 ul sample March 17, 1986	Total ^{32}P Counts in 50 ul sample March 17, 1986	Estimated $^{32}P_{\wedge}$ Counts by Cerenkov March 13, 1986	-5%/day (-20%)	% Recovered Act./Estimated
A	697	17,425	57,186	45,749	38.1
B	369	9,225	30,620	24,496	37.7
C	373	9,325	29,362	23,490	39.7
D	505	12,625	40,864	32,691	38.6

DISCUSSION

The goals of this research project were to generate substrate for processing studies and to use the substrate to detect a 3' terminus processing endonuclease in Sc. pombe. The first of these goals was accomplished by means of a cell-free transcription system. The second goal was not realized during the course of this study. The following factors may have prevented processing of the eluted transcripts:

(i) salts used to precipitate the transcripts may not have been completely removed, and would have inhibited further processing. It seems likely, however, that the two ethanol precipitations, and the washing of the tRNA pellets would have removed the salts. Some traces of salts were nonetheless evident in the microfuge tubes after the samples had been lyophilized. The concentration of these salts in the processing studies may have been sufficient to cause general inhibition of nucleases.

(ii) processing assay conditions were not optimum. It would have been worthwhile to explore the aspects of temperature, time, and enzyme concentration in the processing assay. Processing nucleases were present in the S. cerevisiae enzyme extract, as can be seen by the gel patterns produced from the transcription assay. Failure to extract an active transcription complex from S. cerevisiae unfortunately prevented such studies.

Although detection of the 3' processing endonuclease in Sc. pombe was not accomplished during the course of this research project, continued efforts using the same experimental design should prove fruitful. Generation of substrate (precursor tRNA) is essential in this

study, and the cell-free transcription system provides this substrate in sufficient quantities. Further experimentation on conditions during the S. cerevisiae enzyme extract preparation would need to be undertaken.

The 3' pre-tRNase which was purified from X. laevis ovaries by Castano et al (1985) was shown to accurately process substrate from human and B. mori systems. Unlike the 5' processing endonuclease RNase P, 3' pre-tRNase does not appear to contain an RNA component. It also does not recognize the initial tRNA transcript as substrate, acting rather on 5' processed species.

3' pre-tRNase may be present in yeast systems as well, and may be the enzyme which this investigation had hoped to detect. Because both RNase P and nucleotidyl transferase are found throughout eukaryotic systems, it seems likely that 3' pre-tRNase will also be able to accurately process the 3' terminus of other eukaryotic tRNAs.

Future studies of 3' pre-tRNase should therefore focus on its recognition of other tRNA species, on its presence in a variety of eukaryotic systems, and on its in vivo activities.

BIBLIOGRAPGHY

Adeniyi-Jones, S.; Romeo, P.H.; Zasloff, M. (1984) Nucleic Acids Res. 12: 1101-1115.

Altman, S. Cell (1981) 23: 3-4.

Altman, S.; Bowmann, E.J.; Garber, R.L.; Kole, R.; Koski, R.A.; Stark, B.C. (1980) in tRNA: Biological Aspects (Soll, D.; Abelson, J.; Schimmel, P. eds.); Cold Spring Harbor Press; Cold Spring Harbor, NY: 71-86.

Apirion, D.; Gegenheimer, P. (1984) in Processing of RNA (Apirion, D. ed.); CRC Press, Inc.; Boca Raton, FL: 35-62.

Asha, P.K.; Blouin, R.T.; Zaniewski, R.; Deutscher, M.P. (1983) Proc. Natl. Acad. Sci. 80: 3301-3304.

Bethesda Research Laboratories Catalogue and Reference Guide (1985) Gaithersburg, MD: 134.

Castano, J.G.; Tobian, J.A.; Zasloff, M. (1985) J. Biol. Chem. 260: 9002-9008.

Cooley, L; Appel, B.; Soll, D. (1982) Proc. Natl. Acad. Sci. 79: 6475-6479.

Cooley, L.; Schaack, J.; Burke, D.J.; Thomas, B.; Soll, D. (1984) Mol. Cell. Bio. 4: 2714-2722.

Deutscher, M.P. (1985) Cell 40: 731-732.

Deutscher, M.P. (1984) CRC Crit. Rev. Biochem. 17: 45-71.

Dingermann, T.; Sharp, S.; Appel, B.; DeFranco. D.; Mount, S.; Heiermann, R.; Pongs, O.; Soll, D. (1981) Nucleic Acids Res. 9: 3907-3918.

Freifelder, D. Molecular Biology (1983) Jones & Barlett Publishers, Inc.; Boston: 386-417.

Frendewey, D.; Dingermann, T.; Cooley, L.; Soll, D. (1985) J. Biol. Chem. 260: 449-454.

Garber, R.L.; Gage, L.P. (1979) Cell 18: 817-828.

Greer, C.L.; Peebles, C.L.; Gegenheimer, P.; Abelson, J. (1983) Cell 32: 587-546.

Hopper, A.K. (1984) in Processing of RNA (Apirion, D. ed.); CRC Press, Inc.; Boca Raton, FL: 91-117.

Hottinger-Werken, A.; Schaack, J.; Lapointe, J.; Mao, J.; Nichols, M.; Söll, D. (1985) Nucleic Acids Res. 13: 8739-8747.

Klekamp, M.; Weil, P.A. (1982) J. Biol. Chem. 257: 8432-8441.

Kline, L.; Nishikawa S.; Söll, D. (1981) J. Biol. Chem. 256: 5058-5063.

Kline, L.; Soll, D. (1982) in The Enzymes (Boyer, P.D. ed.); Academic Press; New York: 567-582.

Kole, R.; Altman, S. (1979) Proc. Natl. Acad. Sci. 76: 3795-3799.

Kole, R.; Altman, S. (1982) in The Enzymes , (Boyer, P.D. ed.); Academic Press; New York: 470-483.

Lewin, B. (1985) Genes. Second Edition; John Wiley & Sons; New York: 115-135, 396-404.

Maniatis, T.; Fritsch, E.; Sambrook, J. (1984) Molecular Cloning; Cold Spring Harbor Laboratory; Cold Spring Harbor, NY: 86-94, 247-255.

Orellana, O.; Cooley, L.; Söll, D. (1986) Mol. Cell. Bio., 6: 525-529.

Peebles, C.L.; Gegenheimer, P.; Abelson J. (1983) J. Cell 32: 525-536.

Schaack, J.; Söll, D. (1985) Nucleic Acids Res. 13: 2803-2814.

Schedl, P.; Roberts, J.; Primakoff, P. (1976) Cell 8: 581-585.

Schmidt, O.; Söll, D. (1981) BioScience 31: 34-39.

Schlief, R.F.; Wensink, P.C. (1981) Practical Methods in Molecular Biology; Springer Verlag; New York: 89-125, 173-206.

Sharp, S.J.; Schaack, J.; Cooley, L.; Burke, D.J.; Söll, D. (1985) CRC Crit. Rev. Biochem. 19: 107-144.

Söll, D.; Kline L.K. (1982) in The Enzymes (Boyer, P.D. ed.); Academic Press; New York: 557-566.

Solari, A.; Deutscher, M.P. (1983) Mol. Cell. Biol. 3: 1711-1717.

Zasloff, M.; Santos, T.; Romeo, P.; Rosenberg, M. (1982) J. Biol. Chem. 257: 7857-7863.